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Differential cytotoxicity of clinically important camptothecin derivatives in P-glycoprotein-overexpressing cell lines

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Abstract Camptothecin and its derivatives are specific inhibitors of eukaryotic topoisomerase I (top1) and are active in cancer patients against a variety of refractory solid tumors and leukemia. *Purpose*: The present study further investigated the relationship between multidrug resistance (MDR) mediated by P-glycoprotein^{MDR} and potential resistance to camptothecin derivatives using two experimental systems: (1) MDR KB-V1 cells selected for vinblastine resistance, and (2) NIH3T3 cells transfected with a plasmid expressing wildtype P-glycoprotein^{MDR} multidrug transporter (NIH-MDR-G185). *Results*: We found that both KBV-1 and NIH-MDR-G185 cells were resistant to topotecan, and that topotecan-induced cleavable complexes were reduced in KB-V1 cells, consistent with a role of P-glycoprotein^{MDR} in cellular resistance to topotecan. By contrast, no significant resistance to camptothecin, 9-aminocamptothecin, 10, 11-methylenedioxycamptothecin, or SN-38 (the active metabolite of CPT-11) was observed in NIH-MDR-G185 cells, while KB-V1 cells were cross-resistant to these compounds but produced cleavable complexes similar to those produced by parental KB-3-1 cells. *Conclusions*: These results suggest that topotecan is the only camptothecin tested with significant susceptibility to MDR in cell culture, and that multidrug resistant cells such as KBV1 probably exhibit additional resistance mechanisms to camptothecins besides P-glycoprotein^{MDR} overexpression.

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Introduction

Two water-soluble camptothecin (CPT) derivatives, topotecan (TPT) and CPT-11 (irinotecan), and the water-insoluble derivative, 9-aminocamptothecin (9AC), have recently been introduced clinically. Antitumor activity has been observed in patients with a variety of solid tumors and refractory leukemia [1–5]. CPT and its derivatives are selective topoisomerase I (top1) poisons which trap cleavable complexes by blocking the religation of top1-mediated DNA breaks (for review see references 6–8). The cytotoxicity of CPT derivatives, including SN-38 (7-ethyl-10-hydroxycamptothecin, the active metabolite of CPT-11) and 10,11-methylenedioxycamptothecin (MDO-CPT) [9], has been correlated with cleavable complexes [10, 11], and experimental evidence suggests that cleavable complexes are converted into DNA damage by colliding replication forks (for review see references 6–8). This probably explains why long drug exposures are critical for optimal cytotoxicity in cell culture [12, 13] and clinical activity of CPTs [1–4].

Multidrug resistance (MDR) is a common limitation of cancer chemotherapy. One of its possible causes is overexpression of the P-glycoprotein^{MDR} transporter, an energy-dependent drug efflux pump which prevents cytotoxic drugs such as anthracyclines, epipodophyllotoxins, Vinca alkaloids, and other natural product agents (e.g. taxol) from accumulating intracellularly [14, 15]. Most top2 and tubulin inhibitors are substrates for P-glycoprotein^{MDR}. TPT has also been reported to be a substrate for P-glycoprotein^{MDR} [16–19]. However, the data are limited and sometimes divergent for the other clinically relevant CPT derivatives. For instance, one report states that MDR Chinese hamster CH^{R}C5 cells are tenfold resistant to 9AC [18], while another study

found no significant cross-resistance in human KB-V1 cells [16]. For SN-38, one report indicates tenfold cross-resistance of CH^RC5 cells, while other reports indicate that CPT-11-resistant cells usually have normal drug uptake [20, 21]

The present study was performed to further investigate the relationship between P-glycoprotein^{MDR} and resistance to CPT derivatives using two experimental systems: (1) the MDR, cell line, KB-V1, which was established from the human cervical carcinoma KB cell line as an MDR-expressing clone by stepwise selection with vinblastine [22, 23], and (2) NIH3T3 cells transfected with a plasmid expressing the wildtype P-glycoprotein^{MDR} transporter [24].

Materials and methods

Cell culture

Human carcinoma KB-3-1 and KB-V1 cells were grown in Dulbecco's modified Eagle's medium (ABI, Columbia, Md.) supplemented with 10% fetal bovine serum, 2 m*M* L-glutamine (GIBCO, Gaithersburg, Md.), penicillin (100 units/ml) and streptomycin (100 μg/ml). In order to maintain MDR, KB-V1 were grown in complete medium with 1 μg/ml vinblastine (obtained from the Drug Synthesis and Chemistry Branch at NCI). The doubling times of KB-3–1 and KB-V1 were 24 h and 36–40 h, respectively. Vinblastine was removed from the medium 1 day before drug treatment for KB-V1 cells. Under these conditions, the doubling time of KB-V1 cells was the same as that of KB-3-1 cells (approximately 24 h). NIH-MDR-G185 cells are mouse NIH3T3 cells transfected with a plasmid expressing wildtype P-glycoprotein^{MDR}. These cells were grown in the same medium as the KB cell lines, except that 60 ng/ml colchichine was added to the complete medium to maintain resistance. The colchichine was removed 1 day before cytotoxicity assays. Human leukemia CEM cells were used as internal standard for alkaline elution assays, and were grown in RPMI-1640 medium with 10% fetal bovine serum, 2 m*M* L-glutamine (GIBCO), penicillin (100 units/ml) and streptomycin (100 μg/ml). All cell lines were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

Chemicals

20-*S*-CPT, 9-amino-20-*S*-camptothecin (9AC), TPT and 10, 11-methylenedioxy-20-*S*-camptothecin (MDO-CPT) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, Md.). SN-38 was generously provided by Dr. Terada (Yakult Honsha Co., Tokyo, Japan). Drugs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 m*M* and aliquots were stored at −20 °C.

Clonogenic assays

Exponentially growing KB cells were seeded in 25-cm^2 flasks. Semiconfluent cells were treated with drugs for 8 h, and plated into 25-cm^2 flasks in triplicate (200, 2000 and 20 000 cells/flask). After 7–10 days, colonies were fixed in 95% methanol, stained with 0.05% methylene blue, and counted. Cloning efficiency of both cell lines was 60–70%. Cytotoxicity was calculated as the ratio of cloning efficiency of drug-treated cells to untreated cells. For NIH3T3 cells, exponentially growing cells were seeded into six-well plates (200 cells/well). After attachment, cells were exposed to drugs for 24 h, washed with PBS and incubated in complete medium for 7 days until staining and counting.

DNA damage and cleavable complex assays

Alkaline elutions were carried out as described previously [25–27]. Briefly, KB-3–1 and KB-V1 cells were prelabeled with ^{14}C-thymidine (0.04 μCi/ml), and CEM internal standard cells were prelabeled with ^{3}H-thymidine (0.2 μCi/ml) for 24 h. All cells were chased by resuspension in nonradioactive fresh medium for 20–24 h before drug treatment. Briefly, drug-treated cells were scraped into ice-cold Hanks' balanced salt solution (ABI, Columbia, Md.) containing drug before loading onto the filters. To measure DNA-protein crosslinks, ^{14}C-labeled control cells, drug-treated cells and ^{3}H-labeled internal standard cells (CEM) were irradiated on ice with 30 Gy γ-rays. Irradiated cells were kept on ice and then loaded onto protein-adsorbing filters (Metricel, 0.8 μm pore size, Gelman Sciences Inc., Ann Arbor, Mich.). Cells were lysed with LS10 (2 *M* NaCl, 0.04 *M* Na_2EDTA, 0.2% Sarkosyl, pH 10) and washed with 0.02 *M* EDTA, pH 10. The DNA was eluted with tetrapropylammoniumhydroxide (Pr_4NOH), pH 12.1, without SDS at a flow rate of 0.03–0.04 ml/min. Fractions were collected at 3-h intervals for 15 h.

To measure DNA single-strand breaks, ^{14}C-labeled calibrator cells and ^{3}H-labeled internal standard cells (CEM) were irradiated on ice with 3 Gy. Cells were loaded onto filters (polycarbonate, 2.0 μm, Poretics Corporation, Livermore, Calif.), lysed with SDS-ProK lysis solution [0.1 *M* Glycine, 2% SDS, 0.025 *M* Na_2EDTA, pH 10, 0.5 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, Ind.)] and washed with 0.02 *M* EDTA, pH 10. The DNA was eluted with Pr_4NOH, pH 12.1, containing 0.1% SDS at a flow rate of 0.12–0.16 ml/min. Fractions were collected at 5-min intervals for 30 min. After elution, filters were treated and computation performed as described previously [27, 28].

Northern blot analysis

Human top1 cDNA (T1B) was provided by Dr. W.C. Earnshaw (Johns Hopkins University School of Medicine, Baltimore, Md.) [29]. Human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was purchased from Clontec (Palo Alto, Calif.). Total RNA was extracted with acid guanidium thiocyanate and phenol-chloroform. mRNA was isolated using oligo d(T) beads and analyzed on 1% agarose gels containing 0.66 *M* formaldehyde in 1 × MOPS buffer. Transfer was carried out by capillary action with 10 × SSC to Duralose-UV membrane (Stratagene, La Jolla, Calif.). Fixation of the RNA was accomplished by crosslinking with UV. Hybridization was carried out at 42 °C for 20 h in buffer containing 6 × SSC and 50% formamide. The filter was hybridized with human top1 cDNA probe labeled with α-^{32}P-dATP by a random primer labeling kit (Boehringer Mannheim, Indianapolis, Ind.). Filters were washed twice with 2 × SSC 0.1% SDS and twice with 0.5 × SSC 0.1% SDS at 65 °C. After stripping the top1 probe by boiling for 10 min, filters were rehybridized with a labeled G3PDH cDNA probe as an internal standard. The filters were scanned using a PhosphorImager and quantitated by ImageQuant (Molecular Dynamics, Sunnyvale, Calif.). Top1 expression levels were calculated as the ratio of the top1 band to the corresponding G3PDH band.

Western blot analysis

Antihuman top1 antibodies from the serum of a scleroderma patient were provided by Dr. Earnshaw [30]. Nuclear extracts were prepared from 10^8 cells as previously described [31]. Briefly, cells were harvested and resuspended in ice-cold nucleus buffer (150 m*M* NaCl, 1 m*M* KH_2PO_4, 5 m*M* $MgCl_2$, 1 m*M* ethyleneglycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 0.2 m*M* dithiothreitol, 10% (v/v) glycerol; pH 6.4) containing 1 m*M* 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 20 μg/ml aprotinin. Cell nuclei were isolated by rotating the cells gently with nucleus buffer containing 0.3% Triton X-100 for 30 min at 4 °C. Nuclear extracts were prepared by adding NaCl to 0.35 *M*.

Samples were suspended in the same volume of 2 × loading buffer (0.125 *M* Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue) and separated on a precast 10% polyacrylamide Tris-glycine gel (NOVEX, San Diego, Calif.) in 0.1 *M* Tris base, 0.1 *M* Tricine, 0.1% SDS, pH 8.3. Samples were then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.), and immunoblotting was carried out with the Immuno Select system (GIBCO, Gaithersburg, Md.) and ECL Western Blotting detection reagents (Amersham, Amersham, UK.). The intensity of the top1-specific band was quantitated by computing densitometry and ImageQuant (Molecular Dynamics, Sunnyvale, Calif.).

Results

Cytotoxicity of CPTs in KB-V1 and KB-3-1 cells

Figure 1 shows the cytotoxicity of CPT and its derivatives in KB-V1 and KB-3-1 cells, as measured by clonogenic assays. MDR-expressing KB-V1 cells were more resistant than KB-3-1 cells to all the CPT derivatives. Consistent with previous results obtained in human colon carcinoma HT-29 cells [9, 10], SN-38 and MDO-CPT were the most cytotoxic compounds in the parental KB-3-1 cells, while TPT was less cytotoxic than the other CPT derivatives.

Topoisomerase I-cleavable complexes induced by CPTs in KB-V1 and KB-3-1 cells

Cleavable complexes measure top1 trapped on DNA [6–8]. Thus, if the cross-resistance of KB-V1 cells was due to P-glycoprotein^{MDR}, fewer cleavable complexes would be expected as less drug would reach the top1 target. Drug-induced cleavable complexes were measured in drug-treated cells as DNA–protein crosslinks by alkaline elution [26], and the results are summarized in Fig. 2. TPT induced approximately two-fold fewer DNA protein crosslinks in KB-V1 than in KB cells. This result is consistent with a contribution of P-glycoprotein^{MDR} to cross-resistance of the KB-V1 cells to TPT [16]. By contrast, the other CPT derivatives induced no fewer DNA–protein crosslinks in KB-V1 than in KB-3-1 cells, and SN-38 induced similar a number of DNA–protein

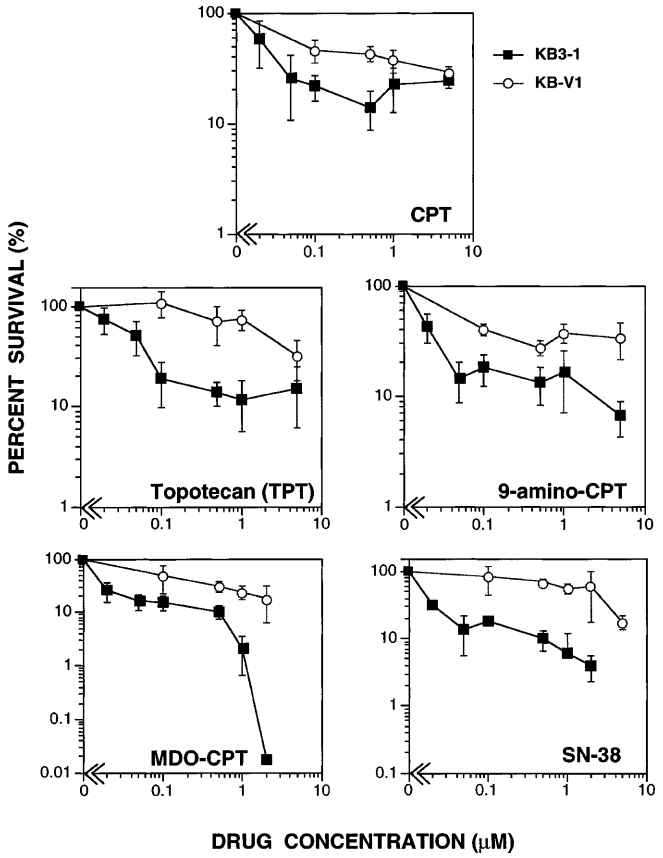

Fig. 1 Cytotoxicity of CPT derivatives in KB-3-1 (■) and KB-V1 (○) cells. Drug treatments were for 8 h, and cytotoxicity was measured by cloning assays. Percent survival was plotted as a function of drug concentration using a log–log scale. Error bars indicate ± SD of at least two independent experiments (*CPT*, camptothecin, *9-amino-CPT* 9-aminocamptothecin, *MDO-CPT* 10,11-methylenedioxycamptothecin, *SN-38* 7-ethyl-10-hydroxycamptothecin

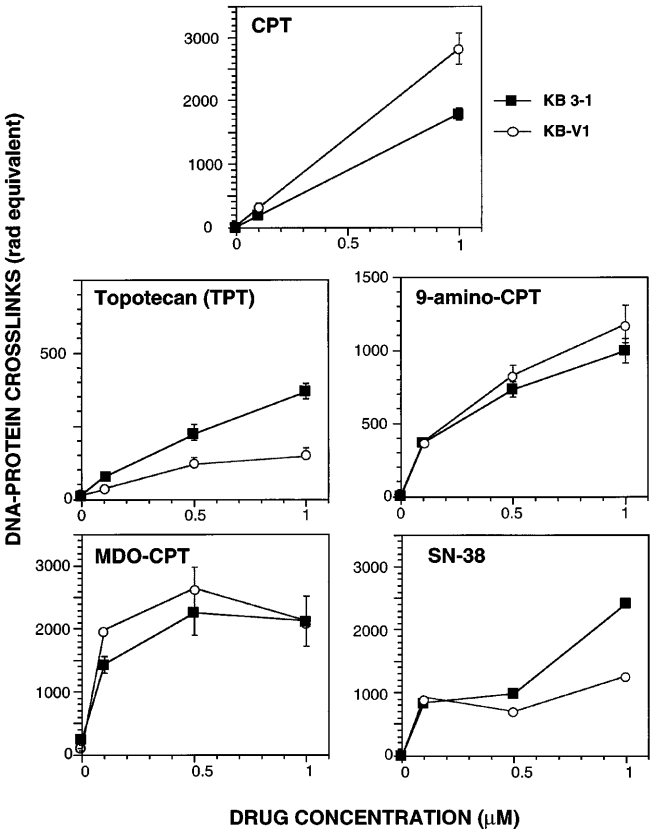

Fig. 2 Drug-induced DNA-protein crosslinks in KB-3-1 (■) and KB-V1 (○) cells. DNA-protein crosslinks were measured by alkaline elution after 1 h drug treatment and are expressed as DNA–protein crosslink rad equivalents. Error bars indicate ± SD of at least two independent experiments. In the case of SN-38, the bars are within the size of the symbols (for abbreviations, see Fig. 1)

Table 1 Time course of DNA single-strand breaks induced by 9AC (1 μ*M*) in KB-3-1 and KB-V1 cells. DNA single strand breaks are expressed as rad-equivalents

Treatment time (h)	KB-3-1	KB-V1
1	1853 ± 250	2057 ± 104
4	1816 ± 240	2140 ± 100
8	1939 ± 22	2388 ± 238

crosslinks in both cell lines at or below 0.5 μM, and fewer DNA–protein crosslinks at 1 μM. These results indicate that resistance of KB-V1 cells to CPT, 9AC, MDO-CPT and to a lesser extent SN-38 is not associated with reduced top1 cleavable complexes. Thus, differences in drug uptake are not responsible for resistance.

The stability of cleavable complexes induced by CPTs was also tested using DNA single-strand break measurements at various times after drug treatment. Cleavable complexes are expected to produce comparable levels of DNA single-strand breaks and DNA–protein crosslinks [26]. Table 1 shows that the trend for 9AC-induced DNA single-strand breaks was also higher in KB-V1 than in KB-3-1 cells, consistent with the DNA–protein crosslink results shown in Fig. 2. DNA single-strand break frequency was within a factor of 2 of the DNA–protein crosslink frequency (as expected for top1-mediated DNA breaks [26]), and single-strand breaks remained unchanged over the whole period of drug treatment. These results further indicate that resistance of KB-V1 cells to 9AC is not associated with fewer cleavable complexes and not related to reduced drug accumulation.

Figure 2 also shows that the CPT derivatives exhibited different potency in inducing DNA–protein crosslinks. MDO-CPT was the most potent derivative, followed by SN-38, 9AC and camptothecin. TPT was the least effective inducer of cleavable complexes. These results are consistent with those obtained in human colon carcinoma HT-29 cells [9, 10, 32] and with purified top1 [33].

Top1 expression in KB-V1 and KB-3-1 cells

Top1 protein levels are commonly decreased in CPT-resistant cell lines including those with well-characterized top1 mutations [21, 34–42]. As indicated in the above sections, this can be explained by CPT and its derivatives turning top1 into a cellular poison that induces DNA damage. Therefore, the lower the level of top1, the lower the DNA damage. Examination of top1 expression in KB-V1 than KB-3-1 cells showed that top1 mRNA and protein levels were not decreased and were even slightly greater (approximately twofold) in KB-V1 than KB-3-1 cells (Fig. 3). These results are consistent with those of Chen et al. in the same cell line [16]. They indicate that reduced sensitivity of KB-V1 cells is not due to reduced top1 expression.

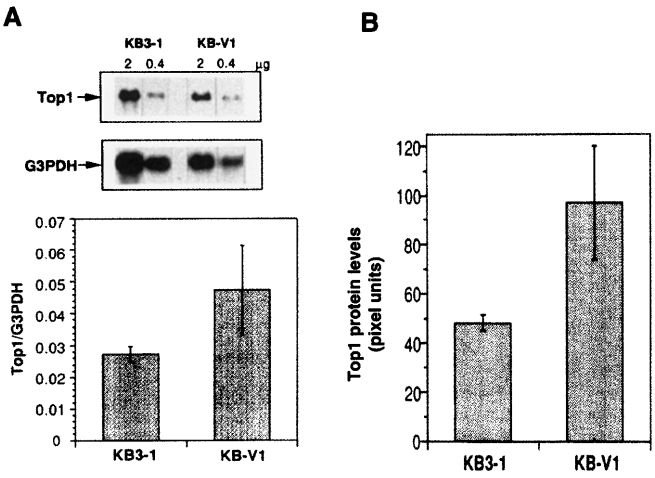

Fig. 3A,B Top1 expression in KB-3-1 and KB-V1 cells. Expression of top1 mRNA (**A**) and protein (**B**) were examined by Northern and Western blotting, respectively. Top1 mRNA levels were measured with a Phosphorimager (Molecular Dynamic, Sunnyvale, Calif.) and expressed as pixel units normalized to that of G3PDH mRNA. Top1 protein levels for similar cell numbers were quantitated in pixel units after scanning Western blots with a densitometer (see Materials and methods)

Cytotoxicity of CPTs in MDR-transfected NIH3T3 cells

The cytotoxicity of CPT derivatives was then compared in an isogenic cellular system. NIH3T3 cells bearing an MDR-expressing construct have recently been described and used to examine the differential effects of P-glycoprotein^{MDR} inhibitors on resistance to different anticancer drugs [24]. We used these cells to elucidate the relationship between P-glycoprotein^{MDR} and cytotoxicity of CPTs. Table 2 shows that significant resistance of NIH-MDR-G185 was only observed for TPT.

Table 2 Cytotoxicity of CPTs in normal and MDR-expressing cells. NIH-MDR-G185 cells express the wildtype (glycine at position 185) MDR gene [24]. Cytotoxicity was determined by clonogenic assays. Treatments were for 24 h. Plating efficiency was 18% to about 40% for NIH3T3 and 40% to about 80% for NIH-MDR-G185 cells (*CPT* camptothecin, *9AC* 9-aminocamptothecin, *MDO-CPT* 10,11-methylenedioxycamptothecin, *TPT* topotecan, *SN-38* 7-ethyl-10-hydroxycamptothecin)

Top1 inhibitors	Relative survival (% control)	
	NIH3T3	NIH-MDR-G185
CPT 0.1 μ*M*	20 ± 18	18 ± 7
TPT 0.1 μ*M*	56 ± 22	95 ± 2
TPT 0.5 μ*M*	6.8 ± 5.5	59 ± 4
TPT 1.0 μ*M*	< 1.0	26.5 ± 6.6
9AC 0.1 μ*M*	28 ± 5	25 ± 10
SN-38 0.1 μ*M*	8 ± 7	13 ± 4
MDO-CPT 0.1 μ*M*	1.2 ± 0.5	1.2 ± 0.5

Discussion

Two conclusions can be drawn from the present study. First, CPT and its clinically important derivatives exhibit different susceptibility to P-glycoprotein^{MDR}-mediated MDR. TPT is the only derivative with unambiguous susceptibility to P-glycoprotein^{MDR} [16–19]. Second, KB-V1 cells probably exhibit additional resistance mechanisms to CPTs besides P-glycoprotein^{MDR} overexpression, as they yield no fewer cleavable complexes than KB-3-1 cells, while being resistant to all the CPTs tested.

The conclusion that TPT is a P-glycoprotein^{MDR} substrate is supported by in vitro studies in P-glycoprotein^{MDR} overexpressing cell lines [16–18] and cell-free systems [19]. However, several points need to be mentioned. First, the in vitro resistance levels to TPT are markedly less than those usually observed with other MDR substrates such as top2 and tubulin inhibitors [14, 15]. Second, MDR tumors in vivo are usually not significantly resistant to TPT [18, 42]. Finally, in contrast to top2 and tubulin inhitors, TPT has not been reported to induce P-glycoprotein^{MDR}. Thus, the therapeutic relevance of P-glycoprotein^{MDR}-mediated resistance remains an open question for TPT.

Two previous studies with 9AC have provided divergent results with respect to P-glycoprotein^{MDR}. Mattern et al. [18] reported that Chinese hamster CH^{R}C5 cells were cross-resistant to 9AC-induced cytotoxicity and cleavable complexes, while Chen et al., [16] using KB-V1 cells, found no cross-resistance using growth inhibition assays. Our results showing that 9AC induced no fewer cleavable complexes in KB-V1 cells and exhibited no cross-resistance in NIH-MDR-G185 are consistent with the view that 9AC is not a P-glycoprotein^{MDR} substrate.

CPT-11 (irinotecan) was initially reported to be active in MDR tumors [43]. However, cross-resistance has been observed in Chinese hamster ovary CH^{R}C5 cells overexpressing P-glycoprotein^{MDR} [18] and in human MCF7/ADR cells [44]. In the latter cells, resistance is actually greater for CPT-11 (14-fold) than for SN-38 (2.4-fold), the active metabolite of CPT-11 [43]. In the present study, KB-V1 cells were also resistant to SN-38, and this resistance was markedly less than for TPT, and NIH-MDR-G185 were not resistant to SN-38, suggesting that SN-38 is not susceptible to P-glycoprotein^{MDR}-mediated resistance. The greater resistance to CPT-11 than SN-38 in some cell lines could be because of a lack of conversion of CPT-11 to SN-38 by carboxyl esterase [21, 39, 45, 46]. This defect can produce cross-resistance to other drugs such as the duocarmycin derivative KW-2189, a recently developed DNA alkylating agent [47]. Increases in intracellular glutathione levels have also been found in CPT-11-resistant glioma cell lines [20]. This may protect cells against a variety of intracellular reactions elicited by anticancer agents and DNA damage.

CPT itself and its more potent derivative, MDO-CPT [9, 33, 48], are probably not P-glycoprotein^{MDR} substrates [16, 19]. Furthermore, CPT does not stimulate P-glycoprotein^{MDR} ATPase [19].

In conclusion, the present studies further demonstrate that the differential spectrum of clinical activity of CPT derivatives against different tumors may be attributed to a number of differences including susceptibility to MDR (in the case of TPT, present study), pharmacokinetics [5] and differential activity against top1 [10, 33].

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